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This project was for continuation of research to investigate the molecular mode of action of a membrane-active hydrocarbon, toluene, potentially present in the Air Force environment as a flight fuel component or from other sources and to serve as a model for other membrane-active molecules in the environment Two important target sites were identified where rapid dose-dependent but reversible changes in membrane organization occurred at low dose levels. One of these was at the plasma membrane where the ability of the membrane to form protuberances was severely compromised. The other concerned a failure to form protuberances by membranes involved in internal trafficking between the endoplasmic reticulum and the Golgi apparatus. This step was reproduced in a cell-free system making detailed studies possible. The toluene inhibited step was identified as dependent on ATP hydrolysis. The involved ATPase activity was characterized, solubilized and partially purified.

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TITLE: A Toluene Model for Hydrocarbon Risk Assessment

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DATE: May 29, 1990

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TECHNICAL REPORT

A. TITLE

A Toluene Model for Hydrocarbon Risk Assessment

B. PRINCIPAL INVESTIGATOR

D. James Morré
Department of Medicinal Chemistry
Purdue University
West Lafayette, IN 47907

C. DATE

May, 1990

D. OBJECTIVES

The proposed research had as its principal objective a detailed investigation of the molecular mode of action of aromatic hydrocarbon, toluene. The findings were anticipated to aid in environmental risk assessment and to serve as a model for other membrane-active agents with potentially similar modes of action.

E. BACKGROUND

The biological action of toluene and related hydrocarbons involves an interaction with membranes. However, to our knowledge there have been no comprehensive studies of the interactions of toluene with biological membranes. Published work has dealt with bioassays and animal studies to establish levels of tolerance or susceptibility and to show that toluene, at extremely high doses, may be disruptive of membrane organization and to cause loss of selective permeability. While the membrane action of toluene may account for the biological responses, there remains a great need for an understanding of toluene in terms of the molecular basis for the membrane alterations as a rational approach to guide risk assessment.

F. SUMMARY OF FINDINGS

The most sensitive cell component to toluene is the plasma membrane where a morphological response in terms of a loss of membrane protuberances and a response in terms of enzymatic activity is observed at 25 ppm both with treatment times of 5 min or less. Thus the plasma membrane is indicated as one important target for toluene intoxication. A perturbation of an ATP or ATPase-dependent reaction also is indicated.

A second target identified was that of the transition region between endoplasmic reticulum and Golgi apparatus where transfer of material appears to be blocked rapidly by 100 ppm or lower toluene both in situ and in a cell free system that was developed to study this phenomenon. Specifically, membranes involved in the internal trafficking between the endoplasmic reticulum and the Golgi apparatus fail to form protuberances. Again perturbation of an ATP- or ATP-dependent step was indicated together with a related involvement of boundary lipids of membrane proteins involved in membrane energization that provide some common denominator between the two different sites of toluene action at the subcellular level.

1) <u>Effects of the sodium ouabain-inhibited Na^+,K^+ ATPase</u>.--Experiments were conducted with the ouabain-inhibited Na^+,K^+ ATPase of liver plasma membranes and cultured cells to show a marked response of this activity in terms of its

sensitivity to ouabain. We have used the activity of a K^+-p-nitrophenylphosphatase (K^+-pNPPase) as a measure of the K^+-stimulated, ouabain inhibited, Na^+ pump ATPase both in situ and with cell fractions. These two activities correlate very closely in other systems. The greater sensitivity of the nitrophenyl substrate make possible activity measurements with smaller amounts of plasma membranes.

Data for rat liver are shown in Figure 1 although similar results have been obtained for cultured cells and membranes from cultured cells as well as from plasma membranes isolated from amphibian epidermis. What we find is that in the presence of very low doses of toluene (maximal response at about 25 ppm), there is a loss of ouabain inhibition. This loss is greatest in the absence of potassium and least in the presence of potassium (Fig. 1). Basal ATPase activity of the membrane preparations is relatively unaffected by toluene as is basal pNPPase activity. The effect is specifically on the ouabain-inhibited component. In fact, in the absence of potassium, ouabain stimulations of the activity are recorded when toluene is present. These findings suggest a specific effect of toluene on the Na^+ pump ATPase of the cell surface perhaps involving a change in the conformation of the activity at or near the ouabain site involved in potassium transport.

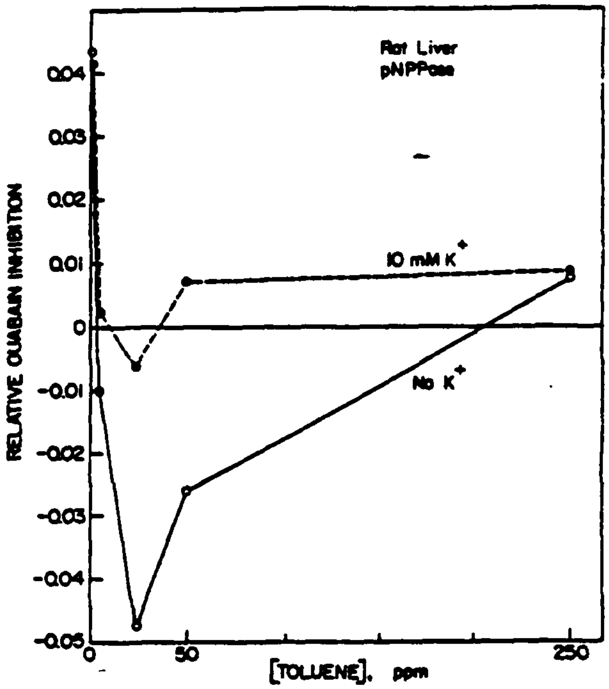

Figure 1. Loss of ouabain inhibition of K^+-stimulated p-nitrophenylphosphatase (pNPPase) of rat liver plasma membranes as a function of toluene concentration.

2) A specific response of endoplasmic reticulum-derived tran-sition elements to toluene.--The basis for the inhibition of membrane flux by toluene in liver slices was the subject of intense investigation. Substantial progress has been made both in the identification of the structural basis for the inhibition and in the formulation of a cell-free system for a detailed molecular study of the basis of toluene action on a specific vesicle formation-fusion process critical to cell growth and development.

a. The structural basis for the response.--An understanding of the structural basis for the toluene response required an appreciation of the complex series of events thought to be involved in the formation of new Golgi apparatus cisternae by the fusion of small 60 nm transition vesicles derived from part-rough (with ribosomes) and part-smooth (lacking ribosomes) elements of the endoplasmic reticulum known collectively as transition elements. This process is illustrated schematically in Figure 2. Blebs form from the endoplasmic reticulum in an energy-requiring process that is aided presumably by the acquisition of a coat protein (not clathrin but perhaps similar to the clathrin coat protein of coated pits and other transport vesicles of endocytosis). The vesicles migrate across the short distance between the endoplasmic reticulum elements and the Golgi apparatus and, at the Golgi apparatus, the small vesicles coalesce to form new Golgi apparatus cisternae. In so doing the vesicles serve as vehicles to transport newly synthesized membranes and perhaps secretory materials as well as from the one compartment to the next in the secretory pathway.

In our experiments with liver slices treated with 500 ppm toluene for varying periods of time, we observed subtle changes in Golgi apparatus/transition element organization, consistent with a specific toluene-induced blockage of the formation the formation of the small endoplasmic-reticulum derived vesicles. These changes were observed subsequently in a variety of test systems including cultured cells where the response was seen at toluene concentrations as low as 100 ppm (Figs. 3 and 4). The overall response illustrated in the figures was a general absence of transition vesicles accompanied by a proliferation (filling in or enlargement) of the existing transition elements associated with the Golgi apparatus as expected if transition element formation were blocked. These morphological observations would explain the findings from the metabolic labeling experiments with liver slices where radioactivity appeared to accumulate in the transition vesicle fraction (ER_0) with an accompanying inhibition of transfer of material to the Golgi apparatus and subsequently to the plasma membrane as well.

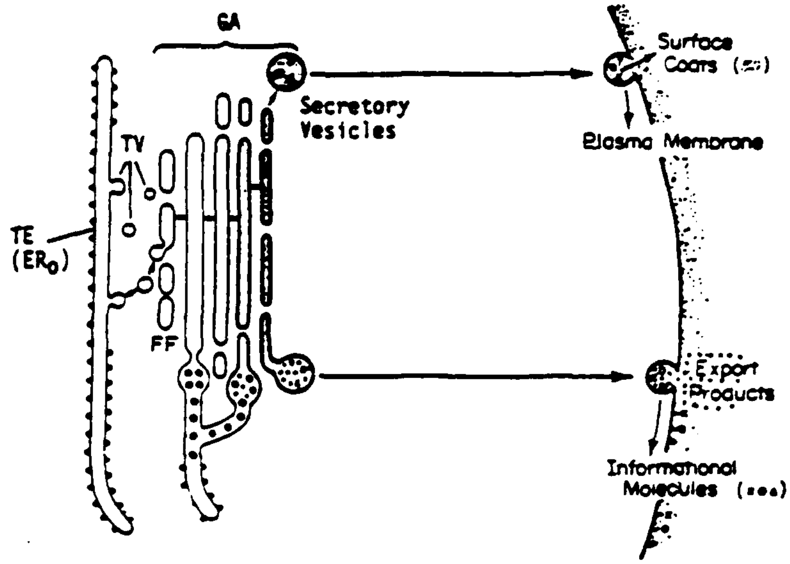

Fig. 2. Diagram illustrating the proposed mechanism for formation of new Golgi apparatus (GA) cisternae by coalescence of transition vesicles (TV) derived from part-rough, part-smooth transition elements (TE = ER_0) of the endoplasmic reticulum.

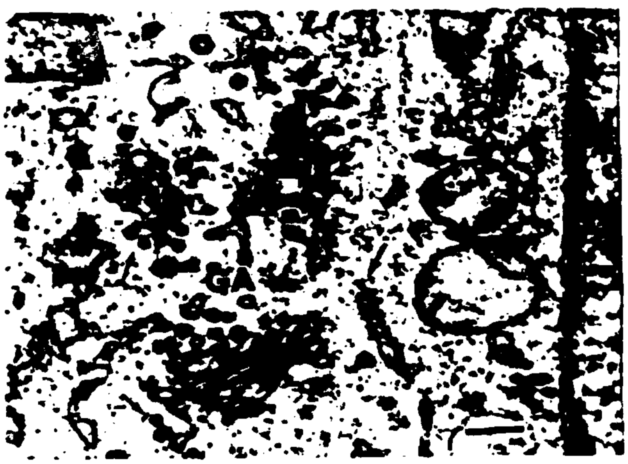

Fig. 3. Electron micrograph of the Golgi apparatus region of a control CHO cell. Note the stacked cisternae of the Golgi apparatus (GA), numerous transition vesicles (small arrows) and the normal appearance of the endoplasmic reticulum elements aligned at the cis Golgi apparatus face (large arrows). Scale bar = 0.2 µm.

Fig. 4. As in Figure 3 but after a brief exposure to 100 ppm tol[illegible]e. The appearance of the Golgi apparatus [illegible] still relatively normal but transition [illegible]esicles are less abundant and the appearance of the Golgi apparatus-associated endoplasmic reticulum is altered in that this membrane system has become much more enlarged and become more elaborate (arrows). Scale bar = 0.2 µm.

b. Development of a cell-free system.--To develop a cell-free system we first isolated from liver and from liver slices a cell fraction enriched in the vesicle forming-transition elements. These fractions when incubated with ATP and a cytosol-derived protein fraction at 37° responded by the production of putative transition vesicles in vitro. In a subsequent series of investigation, we immobilized Golgi apparatus membranes to nitrocellulose strips and demonstrated transfer of material from the transition elements to the Golgi apparatus membranes presumably via the production of transition vesicles. The process was time and temperature dependent and nearly completely blocked by low concentrations (100 ppm) of toluene.

In subsequent studies, we concentrated the transition vesicles formed in vitro by the technique of preparative free-flow electrophoresis (Figs. 5 and 6). In this technique where components to be separated are injected as a fine jet into a separation buffer flowing perpendicular to the field lines of an electric field and membranes having different surface charge densities are readily separated. As illustrated in Figure 5, for a primed transition element system (incubated with ATP, an ATP regenerating system and cytosol), free-flow electrophoresis separates the bulk of the endoplasmic reticulum membranes from a second peak nearer the point of sample injection (less electronegative). This peak consisted of numerous small vesicles, 30% or more with morphologies similar to those of the transition vesicles formed in situ (Fig. 6). They were about 60 nm in diameter with electron

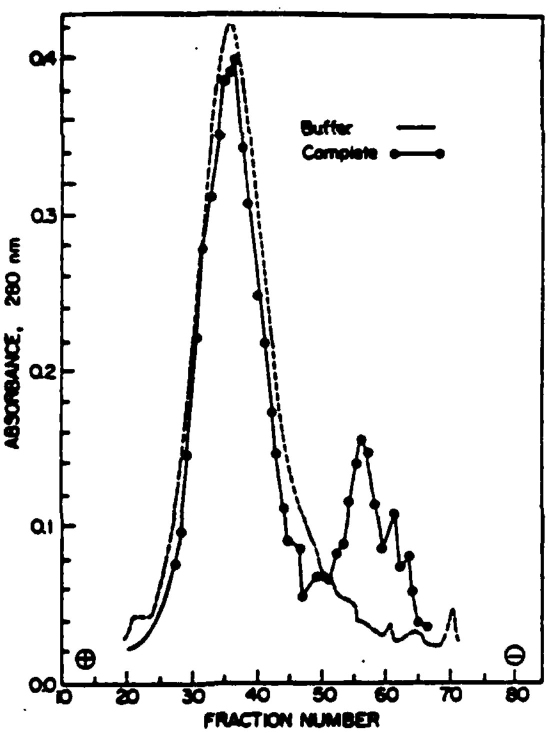

Fig. 5. Free-flow electrophoretic separation of primed (0——0 = complete) and unprimed (---- = buffer only) transition elements of rat liver. The small peak centered at about fraction 56 contained the transition vesicles (see Fig. 6). The major peak centered at fraction 36 contained the bulk of the unaltered starting material.

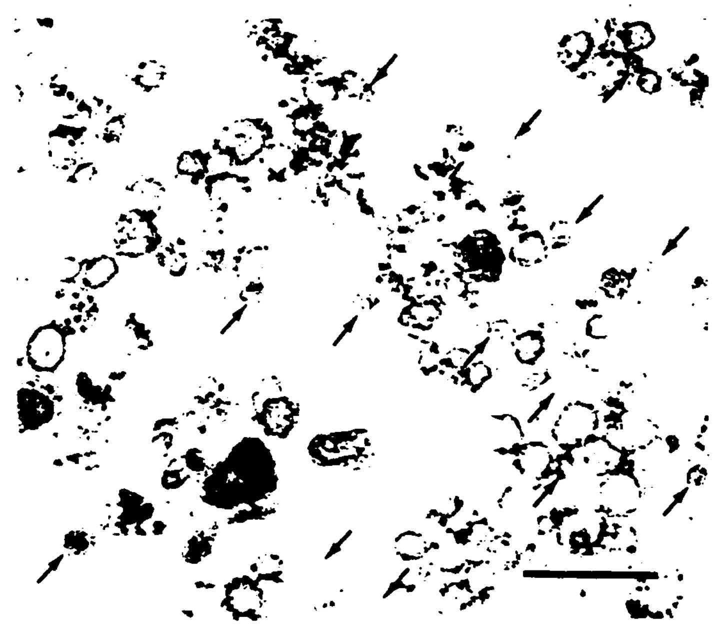

Fig. 6. Electron micrograph of a transition vesicle preparation by preparative free-flow electrophoresis (e.g. right hand peak of Fig. 5). The many 60 nm vesicles with electron dense, nap-like coatings present are indicated by arrows. These preparations containing 30% or more of the membranes present as transition vesicles, were capable of fusing quantitatively with Golgi apparatus preparations immobilized to cellulose nitrate strips. Scale bar = 0.5 μm.

dense and nap-like surfaces. To equate these vesicles functionally to transition vesicles, the free-flow electrophoresis fractions were made radioactive by metabolic labeling of the tissue slices from which they were derived and were added to non-radioactive rat liver Golgi apparatus immobilized to cellulose nitrate strips. Radioactivity was transferred rapidly and quantitatively with a $T_{1/2}$ of less than 5 min. In contrast to vesicle formation, the coalescence of the already-formed vesicles with Golgi apparatus did not require ATP but was enhanced by cytosol and was unaffected by toluene. Thus toluene effects vesicle formation, not fusion, in this system.

The complete incubation mixture was necessary to obtain the formation of transition vesicle fraction illustrated in Figures 5 and 6. The second peak enriched in transition vesicles was present only in preparations incubated with membranes in the complete system, i.e. ATP and ATP regenerating system together with membranes present, only when incubated with ATP plus ATP regenerating system and in cytosol. However, when toluene also was included during the incubation at final concentrations between 100 and 500 ppm, the formation of these vesicles was completely inhibited. These observations corroborate the results observed in vivo and provided the basis for a convenient test system to probe further the molecular details of toluene action in preventing the flux of membranes between endoplasmic reticulum and the cell surface.

3) Characterization of the ATP requiring step.--To determine the nature of the ATP requirement, several non-hydrolyzable ATP analogs such as adenylimidodiphosphate were examined to verify a requirement for ATP hydrolysis. ATP hydrolysis by the vesicle forming transition element in proportion to vesicles formed was shown over the range 0.1 to 20 μM ATP.

pH dependency, nucleoside triphosphate specificity, ion requirements and specificity (Mg^{2+} was added routinely) and response to known inhibitors of enzymatic ATP utilization (vanadate, DCCD, DES) were determined next. ATP hydrolysis by the transitional endoplasmic reticulum fractions was about 50% inhibited by sodium orthovanadate but this compound had no effect on formation of transitional vesicles. The ATPase activity was resistant to N-ethylmaleimide and to nitrate and was not inhibited by oligomycin. Thus, the ATP-driven process was not related to any of the known classes of ATPases. Were it a vacuolar ATPase (V-ATPase), nitrate should have inhibited. Were it a proton ATPase (P-ATPase), vanadate should have inhibited transition vesicle formation. Were it an F-ATPase (mitochondrial type), oligomycin should have inhibited. Only DCCD, which inhibits all known Atpase, was an effective inhibitor.

Next we undertook a series of studies to identify a specific inhibitor of the ATPase that would also inhibit transition vesicle formation in vivo and in vitro. Such an inhibitor was identified in the form of cobalt ions. At a concentration of 50 mM, cobalt ions inhibited the vanadate- and nitrate-resistant ATPase and prevented the formation of transition vesicles from transitional endoplasmic reticulum in vivo as well as in vitro. This is a major new finding resulting from the work that suggests the possibility that the toluene-sensitive ATPase may represent a new class of ATPase hitherto unknown.

4) ATPase purification.--The next step was to isolate and purify the cobalt-sensitive ATPase. Transition elements from liver, were solubilized in detergent and passed over a P-11 phosphocellulose column. The specifically-bound proteins were eluted from the column and further fractionated by preparative polyacrylamide gel electrophoresis. We have succeeded to separate the ATPase from other nucleotide hydrolyzing activities (GTPase, nucleoside diphospatase). However, funds were exhausted before we were able to purify the activity to homogeneity.

5) Summary .

The most sensitive cell component to toluene was the plasma membrane where a morphological response at and a response in terms of enzymatic activity was observed at 25 ppm both with treatment times of 5 min or less. Thus the plasma membrane is indicated as one important target for toluene intoxication. A perturbation of an ATP or ATPase-dependent reaction was indicated.

A second target identified was that of the transition region between endoplasmic reticulum and Golgi apparatus where transfer of material appears to be blocked rapidly by 100 ppm or lower toluene both in situ and in a cell free system newly developed to study this phenomenon. Again perturbation of an ATP- or ATP-dependent step was indicated together with a related involvement of boundary lipids of membrane proteins involved in membrane energization that provide some common denominator between these two points of toluene action at the subcellular level.

The toluene-sensitive ATPase of the transitional endoplasmic reticulum was characterized and partially purified. The results point to its identification as a unique type of ATPase, sensitive to environmental regulation, with specific functions in membrane biogenesis and renewal.